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Evaluation of a Newly Developed HIV Antigen Test

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A new monoclonal antibody-based enzyme immunoassay (Innogenetics) for the detection and quantification of p24 core antigens of HIV-1 (group M and group O) and of HIV-2 was evaluated on 2745 serum samples and 18 culture supernatants and compared with a reference (Coulter) HIV-1 p24 antigen assay. Positive results were confirmed by neutralization with the reagents of the respective tests. As demonstrated by dilution series of HIV cocultures, the new test recognizes p24 antigen of the most common HIV genetic subtypes, including group O and HIV-2. Titres ranged from 729 to 531441. Therefore p24 antigen assay is but very weakly reactive with HIV-2 (titres from 9 to 81).

The new test is considerably more sensitive than the reference. In a population of 365 follow-up samples from 86 different patients, representing all stages of infection, the new test detected p24 antigen at least once in 52% (45/86) of these patients, whereas the reference was positive in 31% (27/86). The newly designed test detected antigen in 40% (145/365) of the samples, while the reference was positive in 21% (75/365). In a group of PCR and/or culture positive neonates, 33% (9/27) of the samples were positive with the new test versus 18% (5/27) with the reference.

The specificity of the new test, as determined on 2,000 blood donor samples, was 99.65% (initially), 99.80% (after repetition), and 100% (with neutralization). The reference scored 99.95%, 100%, and 100%, respectively. In 300 seronegative samples from persons at risk, the initial specificity of the new test was 98.67% (the reference, 99.00%). With neutralization, both assays were 100% specific. ***J. Med. Virol. 53:31–35, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: p24 antigen; HIV; antigen detection

INTRODUCTION

Quantification of p24 antigen human immunodeficiency virus (HIV) can be used for diverse purposes such as the diagnosis of HIV infection, prediction of disease progression and transmission [Erb et al., 1994; Arlievsky et al., 1995], and monitoring the effects of therapeutic antiviral drugs [Goudsmit et al., 1987]. During the first weeks post-infection, a patient is seronegative and diagnosis relies on detection of the viral genome by RT-PCR or antigens by ELISA. Serological diagnosis can be made at 4 to 6 weeks by the detection of HIV antigen, and days to weeks hereafter by the sequential appearance of antibodies to different HIV structural proteins. HIV antigen determination may also be useful in the diagnosis of HIV infection in neonates from seropositive mothers especially in areas where the use of polymerase chain reaction (PCR) is not possible. It has become increasingly clear that in addition to surveillance of circulating CD4+ lymphocyte counts, determination of p24 antigenaemia, and in particular the measurement of viral load by quantification of HIV-1 RNA copy number in plasma, are useful measures of disease progression. HIV antigen quantifitation is useful as an easily measurable marker whose decline indicated the in vivo antiviral activities of anti-HIV agents and is helpful in the early evaluation of new agents [Hammer et al., 1993]. A decline in p24 antigen levels under antiretroviral therapy is associated with a corresponding increase in CD4+ cells [Escaich et al., 1991]. Viral load measurements are also used currently as a sensitive and accurate but expensive alternative for studying progression and drug efficacy.

In the present study, the performance of a newly developed antigen kit of (Innogenetics) was compared with that of the well established Coulter HIV-1 p24 Antigen Assay using a collection of 2745 serum and plasma samples, as well as for a dilution series of 18 HIV and SIVcpz culture supernatants.

MATERIALS AND METHODS

Patients and Samples

In this study the following samples were tested: (i) 2000 serum samples from healthy seronegative blood donors, (ii) 365 follow-up samples from 86 different pa-

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tients with a proven HIV-1 infection, representing samples from diverse stages of the HIV disease [97 Acquired Immunodeficiency Syndrome (AIDS), 69 Persistent General Lymphadenopathy (PGL), 17 AIDS Related Complex (ARC), and 179 asymptomatics], (iii) 80 follow-up samples from 37 different neonates/children [mean age 14 months (23 days–7 years)] born to seropositive women (27 samples were PCR and/or culture positive), (iv) 300 serum samples from seronegative patients at risk, and (v) culture supernatants of 18 different HIV types (8 group M HIV-1 strains from different subtypes: 4 group O, 4 HIV-2, and 2 SIVcpz strains). Threefold dilution series of the culture supernatants were compared with both antigen tests.

The *env* subtypes of group O strains ANT-70 (Vanden Haesevelde et al., 1994), VI686, CA9 [Janssens et al.; in preparation], and MVP5180 [Gürtler et al., 1994] were determined by sequencing and tree construction of the complete *env*-region; for group M strains CA1, CA4, CA5, CA10 [Nkengasong et al., 1994], the sequence of a 900 bp fragment was used; and for the strains VI313, VI824, VI1197, and VI991 the heteroduplex mobility assay (HMA) [Delwart et al., 1993] was employed. For the HIV-2 strains the sequence of the gp 36 (NH_2 terminus) was used for the tree construction [Peeters et al., 1994; Nyambi et al., 1996; Los Alamos database: Meyers et al., 1995].

The *gag* subtype was determined by using the sequence of the p7-fragment for CA1, CA4, CA5, CA10, and a 480 bp fragment for CA9 and VI686. The complete *gag* genome was sequenced for ANT70 and MVP 5180 [Los Alamos database: Myers et al., 1995].

At least one of the following tests was used to prove HIV infection: co-culture with phytohemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMCs) of healthy HIV seronegative human donors [Nkengasong et al., 1995], Western blot HIV Blot 2,2 (Genelabs Diagnostic, Geneva, Switzerland) or New Lav Blot I and II (Sanofi Diagnostics Pasteur), INNO-LIA HIV-1/HIV-2 Ab (Innogenetics, Zwijndrecht, Belgium), or HIV ab (rDNA) p-24 ELISA (Abbott, N. Chicago, IL). PCR for determining HIV infection in neonates and children was performed as described elsewhere [Vandamme et al., 1995]. Each sample was tested with both the new and the reference antigen test.

The Newly Developed HIV Antigen mAb Test

HIV p24 antigen is detected by a solid-phase sandwich-type enzyme-linked immunosorbent assay (EIA). The antigen is trapped between human polyclonal antibodies on the solid phase and murine biotinylated anti-p24 monoclonal antibodies. EIA was carried out according to the manufacturer's instructions. Briefly, 100 μl serum sample were incubated simultaneously with 100 μl conjugate working solution 1 containing biotinylated anti-p24 monoclonal antibodies. After incubation (1 hour at 37°C), the wells were washed 5 times with wash solution. Two hundred μl of conjugate working solution 2 containing peroxidase conjugated streptavidin were added to the wells and incubated for 30 minutes at 37°C. After the washing described above, 200 μl substrate solution (Tetramethyl Benzidin in substrate buffer) were added and the reaction was allowed to proceed for 30 minutes at room temperature before it was stopped with 50 μl stop solution (2N H_2SO_4). Absorbance was read at 450 nm within 15 minutes. Cutoff values were calculated as described by the manufacturer: cutoff = mean OD of the two negative controls +0.050. Signal-to-noise ratios (S/N = OD sample/OD cut off) were calculated for each sample.

Positive samples were treated with the HIV antigen mAb Neutralization Reagents to confirm positivity.

The HIV Antigen mAb Neutralization Reagents

A positive reaction with the new HIV Antigen mAb test was confirmed by a competitive assay. The samples were pre-incubated with a specific neutralizing reagent (human antibodies against p24), followed by the new HIV Antigen mAb procedure. Due to competition between the solid phase antibodies and the antibodies in solution, the serum antigen (if present) is no longer trapped on the plate and no colour development occurs. The wells of the new HIV Antigen mAb assay were filled with 50 μl neutralization solution (NS); and 50 μl of the diluted conjugate solution, containing biotinylated anti-p24 monoclonal antibodies, was added. For each sample, an additional well was filled with 50 μl control solution (CS) and 50 μl conjugate 1 solution. A 100 μl serum sample was added to both wells and the plates were incubated for 30 minutes at 37°C. The plates were further treated as described for the new HIV Antigen mAb assay. An initial positive result was confirmed if a signal reduction (NS well compared to CS well) of more than 50% was observed. A repeated and neutralized reactive test result was considered positive for HIV antigen. False positive samples show a smaller reduction or none.

The Reference HIV-1 p24 Antigen Assay

The HIV antigen test and its neutralization assay were carried out according to the manufacturer's instructions. The cutoff value is the sum of the mean of three negative controls and a predetermined factor of 0.055.

For healthy blood donors, both assays were automated using an RSP 8051 (Tecan, Hombrechtikon, Switzerland) robotic pipettor in line with a BEP 3 (Behringwerke, Marburg, Germany) ELISA processor.

RESULTS

A total of 2,745 serum samples were analysed in parallel using the new and the reference antigen tests.

HIV Negative Serum Samples (Table I)

Of 2,000 blood donor sera, none had a positive screening test for HIV-1/2 antibodies. With the new antigen test, 7 sera were found initially reactive, of which 4 were repeatedly reactive. With the reference, only 1 serum was reactive initially, but no repeatedly. The

TABLE I. Specificity of the Reference HIV-1 p24 Antigen Assay and the New HIV Antigen mAb Test

	Reference HIV-1 p24 antigen assay	New HIV antigen mAb test
Normal blood donors n = 2000		
Initially positive	1 (0.05%)	7 (0.35%)
Repeatedly positive	0 (0%)	4 (0.2%)
Positive after neutralization	0 (0%)	0 (0%)
Samples from patients at risk n = 300		
Initially positive	3 (1%)	5 (1.7%)
Positive after neutralization	0 (0%)	0 (0%)
PCR and culture negative neonates n = 53		
Initially positive	0 (0%)	0 (0%)
Positive after neutralization	0 (0%)	0 (0%)

neutralization reagents identified all reactive samples as false positive. Therefore, when the antigen detection tests are combined with the neutralization assays, both antigen tests showed a 100% specificity.

In a sample of 300 negative sera from individuals at risk for HIV infections 3 and 4 were initially reactive with the reference and the new assays, respectively. None could be neutralized, resulting in 100% specificity for both tests.

Samples From HIV Infected Patients (Table II)

Initially, 155 of 365 follow-up samples of HIV infected individuals were reactive with the new test, and only 81 with the reference. All reference test positive samples were also positive with the new test. After repeat testing (two samples could not be confirmed because of shortage of serum) and neutralization, 145 samples remained positive with the new test. Of 81 samples initially reactive with the reference test, 6 were negative in the control well of the neutralization assay. The remaining 75 samples were confirmed positives by neutralization. Antigen was detected in 45 (52%) and 27 (31%) of 86 HIV infected patients with the new test and the reference, respectively.

Samples From Neonates Born to HIV Antibody Positive Mothers

Twelve of the 27 PCR and/or culture positive samples from HIV infected children were initially reactive with the new test. Nine could be confirmed by neutralization, whereas only 5 were initially reactive in the reference, but these were all confirmed. None of the 53 PCR and culture negative samples from children were positive with either new or reference kits.

At the patient level, 14 patients were HIV infected according to PCR or culture results. Eight remained positive after neutralization. The reference test detected antigen in 4 (35.7%) patients, and all of them were confirmed.

TABLE II. Sensitivity of the Reference HIV-1 p24 Antigen Assay and the New HIV Antigen mAb Test

	Reference HIV-1 p24 antigen assay	New HIV antigen mAb test
Follow-up samples from HIV antibody positive patients n = 365 initially, n = 363 after neutralization		
Initially positive	81 (22.2%)	155 (42.5%)
Positive after neutralization	75 (20.7%)	145 (39.9%)
PCR and/or culture positive neonates n = 27		
Initially positive	5 (18.5%)	12 (44.4%)
Positive after neutralization	5 (18.5%)	9 (33.3%)

Supernatants From Cultures of Different HIV Subtypes (Table III)

Table III compares the sensitivity of the new and reference test using supernatant from cultures of different HIV types and subtypes.

A sensitivity factor is determined as the ratio of the HIV Ag titre (reciprocal of the highest dilution for which the test is still positive) of the new test divided by the Ag titre of the reference test.

The reference was more sensitive than the new test for a single isolate 6 (CA4) belonging to subtype F/D; the optical density (OD) of the last positive dilution was 0.003 OD above the cutoff. For all other isolates belonging to HIV-1 type group M, HIV-1 group O, and SIVcpz, the sensitivity of the new test was higher than or equal to that of the reference. Mean sensitivity factors were 3.9, 6, and 18, respectively.

HIV-2 isolates were detected by the reference only at a dilution endpoint titre of 9 for VI53 and VI1415 and an endpoint titre of 81 for LAV2 and VI884. With the new test, endpoint titres were obtained of 19 683 for VI 53, 59 049 for LAV2, and VI1884, and even an endpoint titre of 177 147 for isolate VI1415.

HIV-2 culture supernatants can be diluted an average of 5831 times more for the new test as compared to the dilution of the reference test.

DISCUSSION

The HIV Antigen mAb (Innogenetics) is a double-sandwich enzyme immuno-assay for the detection of the p24 antigen from HIV in plasma, serum, or viral culture supernatants. A p24 standard curve, which is included in the kit, allows quantification of HIV p24 antigen in the samples. In spiked normal human serum a detection limit of 11 pg p24/ml is reached [Louwagie et al., 1996].

Some authors have shown plasma antigenaemia to be a surrogate marker for disease progression [Ruffault et al., 1995; Lafeuillade et al., 1994; De Gruttola et al., 1994; Hofer, 1994], but the limited sensitivity and dynamic range of the procedure have restricted its use to the subset of patients with high viral loads in whom antigen levels can be followed. In our follow-up study,

TABLE III. Difference in Sensitivity of Two Antigen Tests on Culture Supernatants of Different types and Subtypes of HIV and SIV

Type	Isolate	Name	Subtype Env/Gag	Sensitivity factor[a]
	1	CA1	A/A	9
	2	CA5	B/B	3
	3	VI313	C/A	3
	4	VI824	D/D	1
	5	CA10	A/E	3
	6	CA4	D/F	0.3
HIV-1	7	VI1197	A/G	9
	8	VI991	H/H	3
	9	ANT70	O/O	9
	10	VI686	O/O	3
	11	CA9	O/O	3
	12	MVP5180	O/O	9
HIV-2	13	LAV2	−/A	729
	14	VI53	−/A	2,187
	15	VI884	−/A	726
	16	VI1415	−/A	19,683
SIVcpz	17	SIV cpz-*ant*	—	9
	18	SIV cpz-*gap*	—	27

[a]The ratio of the end point titre of the new test divided by the end point titre of the reference test.
[b]No subtypes have been determined.

we found a 40% positivity rate with Innotest and 21% with Coulter, respectively (Table II). The overall low positivity rate is due to the distribution of clinical stages of the study subjects (97 AIDS, 69 PGL, 17 ARC, and 179 asymptomatics). Others [Jacobson et al., 1995] demonstrated a lack of clinical utility of HIV p24 antigen serum levels (after immune complex dissociation) in patients with asymptomatic HIV disease receiving dideoxynucleoside therapy for early HIV disease. To understand better the biological relevance and potential clinical usefullness of p24 antigen measurements in HIV infection, both the specific antibody levels and non-virion associated antigen should be considered [Brown et al., 1995].

In neonates born to seropositive mothers, the presence of anti-HIV antibodies does not necessarily indicate infection, since antibodies may have been transferred across the placenta. Antigen testing, viral culture, and PCR may contribute to the diagnosis. From 27 PCR and/or culture positive samples, 9 samples were positive with the new and 5 positive with the reference test (Table II). Again, the new test proves to be more sensitive than the reference. None of the PCR and culture negative samples were positive with the new test or with the reference (Table I).

HIV antigen detection in virus culture remains an important tool to monitor virus growth in PHA stimulated human PBMCs of healthy donors. HIV isolation can predict better [Ruffault et al., 1995] therapeutic efficacy of antiretroviral agents in drug trials. The use of RT (reverse transcriptase test) as a mark for HIV production suffers from technical difficulties, lack of specificity, and a long incubation time. The antigen assay is more sensitive, and it has been applied to determine endpoint titrations of infectious HIV in culture for calculations of TCID50 (tissue culture infective dose) and infectivity endpoints to detect antibody neutralization of infectivity.

Although HIV antigen assays are optimised for the detection of p24, which is a conserved region of the genome, some differences occur among the 10 subtypes in group M and group O of HIV-1, HIV-2, and SIVcpz. The new test is capable of detecting a broad range of HIV isolates in supernatants and is more sensitive than the reference (Table III).

The high sensitivity of the new test was also demonstrated in a study with 28 seroconversion panels [Louwagie et al., 1996]. Among the 5 HIV antigen tests evaluated, the new test was the most sensitive. The new test was able to detect HIV antigen earlier (2 cases) or at least coincidently in sera collected serially prior to 10 seroconversions in comparison to 4 other commercial antigen assays. Similarly, continuing antigenaemia after seroconversion was detected later in 9 out of 26 cases in the new test.

Increased detection of HIV antigenaemia can be achieved after immune complex dissociation (ICD) at a low pH by acid treatment of serum samples [Pokriefka et al., 1993] or by simply heating the sample in combination of signal amplification [Schüpbach et al., 1996]. ICD was not investigated in this study. With this approach, good results have been obtained in the diagnosis and prediction in the outcome in developing countries where other diagnostic techniques are not available [Nielsen et al., 1995].

On a sample collection of 2,000 normal human blood donors, new and reference test yielded an initial specificity of 99.65% and 99.95%, respectively. Combining the antigen tests with their respective neutralization procedures resulted in a specificity of 100% for both assays. Out of 300 negative serum samples of patients at risk for HIV infection, initial specificities of 98.67% (new test) and 99% (reference test) were reached. After neutralization, both assays obtained a 100% specificity within this sample collection.

If blood bank screening for HIV antigen is taken into consideration, the reference HIV-1 p24 Antigen Assay has some advantages over the new HIV Antigen mAb in yielding a higher specificity at initial screening. However, the new HIV Antigen mAb test has a greater sensitivity.

In conclusion, the new HIV antigen mAb test from Innogenetics is a more sensitive test than the HIV-1 p24 antigen assay from the reference and is capable of detecting genetically diverse HIV isolates in a faster and more sensitive way. As a consequence of the higher sensitivity rate, the specificity of the new test is slightly lower than the reference test.

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